British Journal of Nutrition (2003), **89**, 259–265
DOI: 10.1079/BJN2002767

The effect of various vitamin E derivatives on the urokinase-plasminogen activator system of ovine macrophages and neutrophils

I. Politis*, A. Voudouri, I. Bizelis and G. Zervas

Department of Animal Science, Agricultural University of Athens, Athens 118 55, Greece

(Received 18 March 2002 – Revised 03 September 2002 – Accepted 22 September 2002)

The effect of vitamin E derivatives on the urokinase-plasminogen activator (u-PA) system of resting and phorbol myristate acetate (PMA)-activated ovine macrophages and neutrophils were investigated. Blood monocyte–macrophages and neutrophils were isolated from twenty-four animals. Macrophages or neutrophils were cultured *in vitro* for 3 or 24 h with or without various vitamin E derivatives: free α-tocopherol (α-T), α-tocopheryl acetate (α-TA), or α-tocopheryl succinate (α-TS). Following incubation, cells were stimulated with 80 μM-PMA. Total cell-associated u-PA, membrane-bound u-PA and free u-PA binding sites were determined before and after stimulation with PMA. Results showed that none of the vitamin E derivatives had any effect ($P>0{\cdot}05$) on the u-PA system of resting monocyte–macrophages or neutrophils. In contrast, α-TS, but not α-TA or α-T, increased ($P<0{\cdot}01$) total cell-associated u-PA and membrane-bound u-PA of PMA-stimulated macrophages and neutrophils. α-TS had no effect ($P>0{\cdot}05$) on total u-PA and membrane-bound u-PA activities of macrophages and neutrophils cultured in the presence of 4-phorbol 12,13 didecanoate, a phorbol ester that does not activate protein kinase (PK) C. Addition of H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), which is a potent inhibitor of both PK A and C, completely abolished the effect of α-TS on total cell-associated u-PA and membrane-bound u-PA of PMA-activated macrophages and neutrophils. Addition of HA1004 (*N*-(2-quanidinoethyl)-5-isoquinoline sulfonamide hydrochloride), which is a potent PK A but a weak PK C inhibitor, had no effect ($P>0{\cdot}05$) on total cell-associated u-PA and membrane-bound u-PA of PMA-activated macrophages and neutrophils cultured in the presence of α-TS. Thus, PK C modulates the effect of α-TS on the u-PA system of ovine macrophages and neutrophils.

α-Tocopherol: Urokinase-plasminogen activator: Protein kinase C

α-Tocopherol (α-T) is a member of the vitamin E group of compounds and possesses extensive biological properties, including potent antioxidant activity (Azzi *et al.* 2000; Bendich, 2001). It functions by inhibiting the expression of nuclear factor-κB, the expression of which is increased during oxidative stress (Yoshikawa & Yoshida, 2000). Furthermore, α-T protects biological membranes of all mammals against lipid peroxidation (De La Fuente & Victor, 2000).

The ability of vitamin E to modulate host immune functions is well established. Vitamin E deficiency has adverse effects on most immune variables, and this is associated with higher rates of infectious diseases and incidence of tumours (Yoshikawa & Yoshida, 2000). Vitamin E supplementation has numerous beneficial effects on the host immune system, including increased proliferative responses of lymphocytes to mitogens, neutrophil chemotaxis and adherence to tissues (De La Fuente & Victor, 2000). A number of studies, reviewed by Azzi *et al.* (2000), established that the effects of vitamin E on macrophage and neutrophil function are exercised through modulation of protein kinase (PK) C activity.

Because of the critical role of vitamin E in the protection of biological membranes and its beneficial effects on the host immune system, supplementation of diets of all animals of importance to agriculture with commercially available vitamin E (α-T) is essential. The most common sources are synthetic all-*rac*-α-T esterified to acetate or to sodium succinate. As regards bioavailability, α-tocopheryl acetate (α-TA) is a better source of vitamin E

Abbreviations: α-T, α-tocopherol; α-TA, α-tocopheryl acetate; α-TS, α-tocopheryl succinate; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; PK, protein kinase; PMA, phorbol myristate acetate; u-PA, urokinase-plasminogen activator.

* **Corresponding author:** Dr I. Politis, fax +30 1 529 4413, email i.politis@aua.gr

than α-tocopheryl succinate (α-TS) in human subjects (Cheeseman *et al.* 1995), sheep (Hidiroglou *et al.* 1992) and broiler chickens (Jensen *et al.* 1999).

Whether all forms of α-T are equally effective in affecting the properties of immunocompetent cells is not known with certainty. Erl *et al.* (1997) reported that α-TS, but not α-TA, inhibits monocytic cell adhesion to endothelial cells. Furthermore, α-TS is a modulator of monocytic differentiation of human monoblasts, as well as a potent inhibitor of cell growth *in vivo* and *in vitro* (Kim *et al.* 1998). Another study established that the α-TS, but not α-TA, inhibited T cell proliferation induced by lectin (Kline & Sanders, 1993).

Migration of neutrophils and macrophages to the site of inflammation is the typical manifestation of the inflammatory response. Neutrophils and macrophages start their migration by penetrating blood capillaries, a process known as diapedesis. To breach the mechanical barriers imposed by the basement membrane, neutrophils may use limited proteolytic activity at regions of cell contact. Neutrophils express a specific urokinase-plasminogen activator (u-PA) receptor (CD 87). Typically, u-PA receptor clusters are observed at the leading edges of migrating cells (Plesner *et al.* 1994). The u-PA can bind to this receptor and, thus, can convert the abundant proteolytically inactive proenzyme plasminogen to active plasmin. Plasmin, in turn, is capable of degrading certain matrix components, in addition to activating other matrix-degrading enzymes such as metalloproteinases (Politis, 2000).

Vitamin E supplementation of dairy cows resulted in enhanced expression of the u-PA gene together with increased activity of membrane-bound u-PA by bovine neutrophils immediately after parturition (Politis *et al.* 2001). Increased membrane-bound u-PA is thought to facilitate the ability of neutrophils to reach the mammary gland after parturition. The mechanism by which vitamin E affects the u-PA system in bovine neutrophils remains elusive. Furthermore, it is not known whether all forms of vitamin E are equally effective in affecting the u-PA system of neutrophils and macrophages.

The objective of the present study was to compare the effects of various α-tocopheryl esters on the dynamics of the u-PA system in resting and PMA-activated ovine macrophages and neutrophils. Whether modulation of PK C is involved in the mechanism by which α-T affects the function of macrophages and neutrophils was examined in detail.

Materials and methods

Animal and leucocyte isolation

Twenty-four lactating sheep of the Chios breed, housed within the premises of the experimental farm of the Agricultural University of Athens, were used to supply blood for macrophage and neutrophil isolation. Each animal was sampled once. Macrophages and neutrophils were isolated using methods previously described by Politis *et al.* (2001). Briefly, 15 ml heparinized (10 IU heparin/ml) venous blood were mixed with 15 ml Hanks' balanced salt solution (HBSS, Sigma Chemical Co., St Louis, MO, USA) and then the mixture was layered onto 20 ml sodium metrizoate (95 g/l)–polysaccharide (56 g/l) gradient with a specific gravity of $1{\cdot}077 \times 10^6$ g/m^3 (Sigma Chemical Co.) and was centrifuged at 500 ***g*** for 45 min.

For isolation of monocytes, cells from the interface were collected, washed twice in RPMI-1640 (Sigma Chemical Co.) and resuspended in RPMI-1640 containing fetal bovine serum (100 ml/l) at a concentration of 1×10^7 cells/ml. For neutrophil isolation, cells from the bottom layer were washed twice in RPMI-1640 medium and the erythrocytes were lysed by addition of 20 ml sterile distilled water, followed by addition of 10 ml sterile saline solution (27 g NaCl/l) to restore isotonicity. Cells were pelleted by centrifugation (200 ***g***, 10 min) and resuspended in RPMI-1640 medium containing fetal bovine serum (100 ml/l) at a concentration of 1×10^7 cells/ml. Cell viability was assessed by Trypan blue dye exclusion (Sigma Chemical Co.) and was always high (>95 %). The purity of the isolated monocytic and neutrophil cell populations was always >95 %.

Vitamin E treatment

Cells (monocytes or neutrophils; 2×10^6) were resuspended in 1 ml RPMI-1640 containing fetal bovine serum (100 ml/l) in the absence (control) or presence of various vitamin E derivatives: α-T (0–100 μM), α-TA (0–100 μM) or α-TS (0–100 μM) and were incubated for 3 or 24 h. All vitamin forms were purchased from Sigma Chemical Co. and were dissolved in ethanol before addition in the medium in the final concentrations mentioned earlier, ensuring that the maximum amount of ethanol in the medium would be <1 ml/l. For control purposes, cells (2×10^6) were resuspended in 1 ml RPMI-1640 containing fetal bovine serum (100 ml/l) ethanol (1 ml/l) or ethanol (1 ml/l) plus succinate (0–25 μM; Sigma Chemical Co.) and were maintained for 3 or 24 h. Following incubation, cells were recovered, washed three times in RPMI-1640 and processed further as described later.

Determination of urokinase-plasminogen activator activity

Total cell-associated urokinase-plasminogen activator activity. Total cell-associated u-PA activity (intracellular + membrane-bound) was measured in resting and activated monocyte–macrophages and neutrophils. Macrophages and neutrophils were activated following treatment with 80 μM-phorbol myristate acetate (PMA) for 30 min. This combination of concentration and time is sufficient to induce optimal stimulation of neutrophils and macrophages (Politis *et al.* 2001).

Following treatment for 3 h with various derivatives of vitamin E, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and were resuspended in 500 μl HBSS containing 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (Sigma Chemical Co.) with or without 80 μM-PMA (Sigma Chemical Co.). After incubation for 30 min at 37°C, cells were washed three times with HBSS. Cells were then lysed by addition of 500 μl 1 mM-sodium bicarbonate, centrifuged at 12 000 ***g*** for 3 min, divided into

portions, and stored at −80°C. Activities of u-PA in portions of lysed neutrophils or macrophages were determined following the procedure described by Politis *et al.* (2001). Briefly, reactions were performed in a total volume of 250 μl 100 mM-Tris buffer (pH 8·0) containing 100 mM-NaCl, 50 μg plasminogen/ml (Sigma Chemical Co.), 0·6 mM-valine–leucine–lysine–*p*-nitroanilide (Sigma Chemical Co.) and 10 μl lysed cells. The reaction mixture was incubated for up to 3 h and absorbance at 405 nm was measured at 15 min intervals using a microtitre-plate reader. The rate of *p*-nitroaniline formation was calculated from the linear part of the curve for absorbance *v.* time. A sample without plasminogen served as a control.

The assay system utilizes the enzymatically active u-PA present within the lysed neutrophils to convert exogenously supplied plasminogen to active plasmin. Plasmin, so produced, is subsequently allowed to attack the chromogenic substrate valine–leucine–lysine–*p*-nitroaniline adjacent to lysine and liberate the free chromophore *p*-nitroaniline. In this system, changes in colour are directly related to plasmin concentrations, and, therefore, indirectly to u-PA activity.

Membrane-bound urokinase-plasminogen activator activity. Membrane-bound u-PA is catalytically active and, thus, its activity can be measured in 'live' PMA-stimulated and resting monocyte–macrophages or neutrophils (Politis *et al.* 2001). Following treatment for 3 h with various derivatives of vitamin E, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and were resuspended in 500 μl HBSS containing 20 mM-HEPES with or without 80 μM-PMA. After incubation for 30 min at 37°C, cells were washed three times with HBSS and finally resuspended at various cell concentrations in 250 μl 100 mM-Tris buffer (pH 8·0). Membrane-bound u-PA was determined following the procedure described in detail by Politis *et al.* (2001).

To eliminate the possibility that some u-PA was secreted in the medium, in control wells, monocyte–macrophages or neutrophils were removed by centrifugation before each determination. No detectable activity was found in the medium in which macrophages and neutrophils were cultured. This indicates that no u-PA was released during the incubation period. Therefore, any changes in colour are due to conversion of plasminogen to plasmin by catalytically active u-PA present on cell membrane. Cells remained viable and intact throughout this time period.

Free urokinase-plasminogen activator binding sites on cellular membranes. The u-PA binding sites present on the cell membrane of monocyte–macrophages or neutrophils may or may not be fully saturated (Plesner *et al.* 1994). The free, unoccupied u-PA binding sites can be fully saturated following incubation of monocyte–macrophages or neutrophils with purified u-PA. Thus, the difference in membrane-bound u-PA before and after incubation with u-PA reflects the presence of free u-PA binding sites on the cell membrane of macrophages or neutrophils.

Following treatment for 3 h with various derivatives of vitamin E, monocyte–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and were resuspended in 500 μl HBSS containing 20 mM-HEPES with or without 80 μM-PMA. After incubation for 30 min at 37°C, cells were washed three times with HBSS and resuspended in 500 μl HBSS plus 20 mM-HEPES containing purified u-PA (Sigma Chemical Co.; 10 units/ml). After incubation for another 30 min at 37°C, cells were washed three times with HBSS, and then resuspended at various cell concentrations in 250 μl 100 mM-Tris buffer (pH 8·0) for the determination of the membrane-bound u-PA as described earlier. Incubation of macrophages or neutrophils for 30 min at 37°C in the presence of u-PA is sufficient to fully saturate all u-PA binding sites on the cell membrane (Politis *et al.* 2001).

The specificity of the binding of the exogenous u-PA on the cell membranes was verified using the competition assays described in detail by Politis (1996).

Assessment of the protein kinase C involvement

Following treatment for 3 h with or without α-TS (25 μM), monocytes–macrophages or neutrophils (2×10^6/ml) were washed three times with HBSS and were resuspended in 500 μl HBSS containing 20 mM-HEPES, 80 μM-PMA, 0–100 μM-H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; Sigma Chemical Co.) or 0–500 μM-HA1004 (*N*-(2-quanidinoethyl)-5-isoquinoline sulfonamide hydrochloride; Sigma Chemical Co.). H7 is a potent inhibitor of both PK A and C (Hidaka *et al.* 1984) while HA1004 is a potent PK A but a weak PK C inhibitor (Asano & Hidaka, 1984). After incubation for 30 min at 37°C, cells were washed, recovered and total cell-associated u-PA and membrane-bound u-PA activities were determined as described earlier.

In another experiment, macrophages or neutrophils were stimulated with 80 μM 4-phorbol 12,13 didecanoate instead of PMA. This compound belongs to the family of phorbol esters but does not activate PK C (Lowe *et al.* 1992). Total cell-associated u-PA and membrane-bound u-PA activities were determined as described earlier.

Statistical analysis

Values are reported as means and standard deviations. Differences between the means were determined using Student's *t* test.

Results

Resting macrophages and neutrophils

Low total cell-associated (0·026–0·055 Δ*A*/h) or membrane-bound (0·025–0·055 Δ*A*/h) u-PA activities were detected in resting monocyte–macrophages and neutrophils. The majority of the cell-associated u-PA (>90 %) is present on the plasma membranes of both monocyte–macrophages and neutrophils. To examine whether monocyte–macrophages and neutrophils have free, unoccupied u-PA binding sites in their plasma membranes, cells were incubated for 30 min in the presence of purified u-PA (10 IU/ml medium per 2×10^6 cells). Results showed that incubation of cells with u-PA led to dramatic increases of 16·4- and 12·5-fold ($P < 0·01$) in membrane-bound u-PA activity of macrophages and neutrophils respectively. This suggests

Table 1. Effects of various vitamin E derivatives on total cell-associated urokinase plasminogen activator (u-PA) and membrane-bound u-PA activities and free u-PA binding sites of ovine macrophages activated by phorbol myristate acetate (80 μM)†

(Mean values and standard deviations for twenty-four observations per group)

	u-PA activity (Δ*A*/h)					
	Total		Membrane-bound		Free u-PA binding sites	
Treatment	Mean	SD	Mean	SD	Mean	SD
Control	0·340	0·151	0·340	0·140	0·440	0·130
α-T (25 μM)	0·365	0·172	0·357	0·156	0·442	0·147
α-TA (25 μM)	0·360	0·157	0·358	0·159	0·435	0·152
α-TS (25 μM)	0·452**	0·113	0·430**	0·125	0·361**	0·137

α-T, α-tocopherol; α-TA, α-tocopheryl acetate; α-TS, α-tocopheryl succinate.
Mean values were significantly different from those of the control group: ** $P \leq 0{\cdot}01$.
† For details of procedures, see p. 260.

the presence of numerous free, unoccupied u-PA binding sites on the cell membrane of both cell types; this finding is in agreement with previous reports regarding bovine macrophages and neutrophils (Politis *et al.* 2001).

The effect of various vitamin E derivatives on total cell-associated u-PA and membrane-bound u-PA activities and free, unoccupied u-PA binding sites on cell membrane of resting monocyte–macrophages and neutrophils was examined. None of the vitamin E derivatives (α-T, α-TS, α-TA) had any effect ($P > 0{\cdot}05$) on total cell-associated u-PA and membrane-bound u-PA activities or the free, unoccupied u-PA binding sites of monocyte–macrophages and neutrophils (results not shown).

Activated macrophages and neutrophils

α-TS, but not α-T or α-TA, caused increases ($P < 0{\cdot}01$) in total cell-associated u-PA and membrane-bound u-PA activity and a decrease in free, unoccupied u-PA binding sites of macrophages (Table 1) and neutrophils (Table 2) compared with control values. The majority of the cell-associated u-PA in PMA-activated macrophages and neutrophils was present on the plasma membranes. As expected, PMA, a known activator of the u-PA system in human and bovine macrophages and neutrophils, caused dramatic increases ($P < 0{\cdot}01$) in total cell-associated u-PA and membrane-bound u-PA activities of both macrophages and neutrophils compared with corresponding values of resting cells (0·025–0·055 Δ*A*/h). However, PMA decreased the free, unoccupied u-PA binding sites of neutrophils by 35–50 % ($P < 0{\cdot}01$), but had no effect ($P > 0{\cdot}05$) on that of macrophages compared with values of the corresponding resting cells.

To ensure the specificity of the α-TS effect, a number of experiments were performed and the results are summarized in Table 3. These experiments showed that the effect of α-TS on total cell-associated u-PA and membrane-bound u-PA activities of macrophages and neutrophils were dose-related (Table 4). The optimal stimulation of u-PA in macrophages and neutrophils was observed when these cells were cultured in the presence of 25 μM-α-TS. Total cell-associated u-PA activities of macrophages and neutrophils cultured in the presence of α-TS for 3 h were similar ($P > 0{\cdot}05$) to those of the corresponding cells cultured in the presence of α-TS for 24 h (Table 4).

In other experiments, we tested whether the effect of α-TS on the u-PA system of activated macrophages and neutrophils could be altered (up regulated or down

Table 2. Effects of various vitamin E derivatives on total cell-associated urokinase plasminogen activator (u-PA) and membrane-bound u-PA activities and free u-PA binding sites of ovine neutrophils activated by phorbol myristate acetate (80 μM)†

(Mean values and standard deviations for 24 observations per group)

	u-PA activity (Δ*A*/h)					
	Total		Membrane-bound		Free u-PA binding sites	
Treatment	Mean	SD	Mean	SD	Mean	SD
Control	0·450	0·170	0·402	0·104	0·329	0·100
α-T (25 μM)	0·456	0·160	0·452	0·167	0·395	0·166
α-TA (25 μM)	0·446	0·165	0·452	0·154	0·394	0·165
α-TS (25 μM)	0·619**	0·175	0·610**	0·163	0·297**	0·133

α-T, α-tocopherol; α-TA, α-tocopheryl acetate; α-TS, α-tocopheryl succinate.
Mean values were significantly different from those of the control group: ** $P \leq 0{\cdot}01$.
† For details of procedures, see p. 260.

Table 3. Effects of various concentrations and incubation times (3 or 24 h) of α-tocopheryl succinate (α-TS) or succinate alone on total cell-associated urokinase-plasminogen activator (u-PA) activities of ovine macrophages and neutrophils activated by phorbol myristate acetate (80 μM)†

(Mean values and standard deviations for twelve observations per group)

	u-PA activity (Δ*A*/h)							
	3 h				24 h			
	Macrophages		Neutrophils		Macrophages		Neutrophils	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
α-TS (μM)								
0	0·439	0·104	0·328	0·102	0·422	0·108	0·317	0·088
10	0·477	0·186	0·357	0·167	0·471	0·113	0·353	0·097
25	0·626**	0·101	0·471**	0·103	0·617**	0·107	0·468**	0·078
100	0·616**	0·114	0·470**	0·126	0·620**	0·122	0·475	0·102
Succinate (μM)								
25	0·442	0·108	0·325	0·105	0·709	0·106	0·341	0·090

Mean values were significantly different from those of the control group (0 μM α-TS): ** $P \leq 0{\cdot}01$.
† For details of procedures, see p. 260.

regulated) when cells were incubated with α-TS for longer incubation periods (48 and 72 h). α-TS was equally effective for all longer incubation times tested (24–72 h) (results not shown). Therefore, all experiments were performed using the shorter effective incubation time (3 h), with the expectation that after short incubation the cells will have properties closer to those in the *in vivo* situation than cells after longer incubation periods.

We consider it possible that the effect of α-TS on the u-PA system of macrophages and neutrophils may be due to the succinate moiety of the α-TS molecule. Against this notion, succinate alone had no effect ($P > 0{\cdot}05$) on total cell-associated u-PA activities of macrophages and neutrophils incubated with α-TS for 3 or 24 h (Table 3).

Protein kinase C involvement

The effect of two different PK inhibitors on total cell-associated u-PA activities of PMA-activated macrophages and neutrophils were examined. Addition of H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), which is a potent inhibitor of both PK A and C, completely abolished the effect of PMA on total cell-associated u-PA and membrane-bound u-PA activities of macrophages and neutrophils cultured with or without α-TS (Table 5). Addition of HA1004 (*N*-(2-quanidoethyl)-5-isoquinoline sulfonamide hydrochloride), (a potent PK A but a weak PK C inhibitor) in a wide range of concentrations (0–500 μM) had no effect ($P > 0{\cdot}05$) on total cell-associated u-PA and membrane-bound u-PA activities of macrophages and neutrophils cultured with or without α-TS (Table 5).

To examine the role of PK C further, the effect of 4-phorbol 12,13 didecanoate, a phorbol ester that does not activate PK C, on total cell-associated u-PA and membrane-bound u-PA activities of monocyte–macrophages and neutrophils was examined. Addition of 4-phorbol 12,13 didecanoate alone or with α-TS (200 μM) had no effect ($P > 0{\cdot}05$) on total cell-associated u-PA and membrane-bound u-PA activities of macrophages and neutrophils compared with the corresponding values of the resting cells (Table 4).

Table 4. Effects of α-tocopheryl succinate (α-TS; 200 μM) on total cell-associated urokinase-plasminogen activator (u-PA) activities of 4-phorbol 12,13 didecanoate (PDDA; 80 μM)-treated ovine macrophages and neutrophils*

(Mean values and standard deviations for eight observations per group)

	u-PA activity (Δ*A*/h)							
	Macrophages				Neutrophils			
	Total		Membrane-bound		Total		Membrane-bound	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0·028	0·007	0·026	0·007	0·051	0·010	0·049	0·010
PDDA	0·024	0·010	0·026	0·010	0·055	0·019	0·060	0·026
PDDA+α-TS	0·027	0·010	0·024	0·010	0·050	0·012	0·055	0·012

* For details of procedures, see p. 260.

Table 5. Effect of two protein kinase inhibitors on total cell-associated urokinase plasminogen activator (u-PA) and membrane-bound u-PA activities of phorbol myristate acetate (PMA, 80 μM)-activated ovine macrophages and neutrophils cultured in the presence or absence of α-tocopheryl succinate (α-TS 25 μM)‡

(Mean values and standard deviations for twelve observations per group)

	u-PA activity (Δ*A*/h)							
	Macrophages				Neutrophils			
	Total		Membrane-bound		Total		Membrane-bound	
Treatment	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Control	0·031	0·014	0·028	0·009	0·052	0·010	0·048	0·010
PMA	0·355**	0·055	0·340**	0·045	0·465**	0·136	0·448**	0·108
PMA + H7	0·049	0·009	0·045	0·006	0·107	0·041	0·101	0·037
PMA + HA1004	0·356**	0·058	0·358**	0·062	0·466**	0·093	0·451**	0·105
PMA + α-TS	0·484††	0·086	0·470††	0·069	0·644††	0·086	0·607††	0·193
PMA + α-TS + H7	0·047	0·006	0·048	0·021	0·101	0·026	0·095	0·021
PMA + α-TS + HA1004	0·463††	0·054	0·479††	0·062	0·617††	0·126	0·592††	0·144

H7, 1-(5-isoquinolinesulfonyl)-2-methyl piperazine dihydrochloride (100 μM); HA1004, *N*-(2-quanidinoethyl)-5-isoquinoline sulfonamide hydrochloride (100 μM).
Mean values were significantly different from those of the control group: ** $P \leq 0{\cdot}01$.
Mean values were significantly different from those of the PMA-treated group †† $P \leq 0{\cdot}01$.
‡ For details of procedures, see p. 260.

Discussion

The first finding emerging from the present study is that the α-TS enhanced total cell-associated u-PA and membrane-bound u-PA activities of PMA-activated ovine macrophages and neutrophils. Three lines of evidence suggest that the induction of the u-PA system is a unique property of α-TS. First, the two other forms tested (α-T and α-TA) had no effect on total cell-associated u-PA and membrane-bound u-PA activities of PMA-activated ovine macrophages and neutrophils. Second, succinate alone had no effect on the u-PA system of resting and PMA-activated ovine macrophages and neutrophils. Third, addition of α-TS to *in vitro* cultures of ovine macrophages and neutrophils affected the u-PA system in a dose-dependent manner. In fact, optimal stimulation of ovine macrophages and neutrophils was observed when these cells were cultured in the presence of 25 μM-α-TS, which is a relatively high vitamin E concentration, but can be encountered in physiological situations (Politis *et al.* 2001).

The second finding emerging from the present study is that the effect of the α-TS on the u-PA system of ovine macrophages and neutrophils requires activation of the PK C system. This conclusion is supported by three important observations. First, α-TS was ineffective towards resting (not PMA-treated) ovine macrophage and neutrophils. PMA is a known activator of the PK C system (Plesner *et al.* 1994; Politis *et al.* 2001). Second, α-TS, used at a very high concentration (200 μM), had no effect on the u-PA system of ovine macrophages and neutrophils when PMA was substituted by 4-phorbol 12,13 didecanoate, a phorbol ester that does not activate PK C (Lowe *et al.* 1992). Third, addition of H7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride), which is a potent inhibitor of both PK A and C (Hidaka *et al.* 1984), completely abolished the effect of α-TS on the u-PA system of PMA-stimulated ovine macrophages and neutrophils. Based on the H7 results, PK C or A or both are implicated in the mechanism through which α-TS affects the u-PA system. On the other hand, addition of HA1004 (*N*-(2-quanidoethyl)-5-isoquinoline sulfonamide hydrochloride), which is a potent PK A but a weak PK C inhibitor (Asano & Hidaka, 1984), used in a wide range of concentrations (0–500 μM), had no effect on the u-PA system of PMA-stimulated ovine macrophages and neutrophils. Thus, the PK C, and not the PK A system, is implicated in the mechanism through which α-TS affects the u-PA system.

The work of others, reviewed by Azzi *et al.* (2000), suggested that numerous effects of α-T on several cell types are exercised through modulation of the PK C system. There is, however, an important difference between the present and previous studies. We found that α-TS enhanced the effect of PMA on the u-PA system of macrophages and neutrophils; the effect of PMA is exercised through the PK C system (Plesner *et al.* 1994; Politis *et al.* 2001). Others have shown that α-T inhibited the PK C system; Devaraj *et al.* (1996) and Kanno *et al.* (1996) showed that α-T blocked superoxide production and cell adhesion by human PMA-stimulated macrophages and neutrophils. Others have demonstrated that α-T inhibits respiratory burst in human macrophages via a mechanism involving PK C inhibition, followed by attenuation of p47 (phox) phosphorylation and membrane translocation (Cachia *et al.* 1998).

Previous studies have shown that the α-TS affected the expression of various cell surface proteins of monocytes-macrophages; the effect is dependent on the differentiation state of these cells. Kim *et al.* (1998) showed that α-TS, but not α-TA, enhanced expression of CD11c and integrins α5 and β1 by the U937 promonocytic cells. Monocytic differentiation is associated with expression of the proteins mentioned earlier on the cell membrane and the presence of these proteins is required for interaction of monocytes–macrophages with various extracellular proteins. On the other hand, α-TS did not affect the expression of CD11c

and integrins α5 and β1 in terminally differentiated macrophages (Kim *et al.* 1998). The ability of α-TS to affect expression of various cell surface proteins by monocytes–macrophages might be species-specific and dependent upon the differentiation state of these cells.

Why is it that α-TS, but not α-TA, has an effect on the u-PA system of ovine PMA-activated macrophages and neutrophils? α-TS is more hydrophilic compared with the other two forms (α-T, α-TA) used in the present study. The physical and chemical conditions involved in the interaction of various α-T forms with ovine macrophages and neutrophils might make it easier for the α-TS to be absorbed by these cells. Rego *et al.* (1998) reported higher intracellular α-T levels when retinal cells were cultured in the presence of α-TS compared with α-TA. Whether α-T is preferentially absorbed by ovine macrophages and neutrophils when these cells are cultured in the presence of α-TS compared with α-TA and the free alcohol form will be the topic of our future studies.

In summary, the present experiments showed that the α-TS, but not α-TA or the free alcohol form, enhanced total cell-associated u-PA and membrane-bound u-PA by PMA-activated ovine macrophages and neutrophils. Furthermore, the effect of α-TS on ovine cells was exercised through modulation of the PK C system.

References

Asano T & Hidaka H (1984) Vasodilatory action of HA 1004 [N-(2-quanidinoethyl)-5-isoquinoline sulfonamide hydrochloride], a novel calcium antagonist with no effect on cardiac function. *Journal of Pharmacology and Experimental Therapeutics* **231**, 141–145.

Azzi A, Breyer I, Feher M, Pastori M, Ricciarelli R, Spycher S, Staffieri M, Stocker A, Zimmer S & Zingg JM (2000) Specific cellular responses to α-tocopherol. *Journal of Nutrition* **130**, 1649–1652.

Bendich A (2001) Micronutrients in women's health and immune function. *Nutrition* **17**, 858–867.

Cachia O, El Benna J, Pedruzzi E, Descomps B, Gougerot-Podicalo MA & Leger CL (1998) α-Tocopherol inhibits the respiratory burst in human monocytes – attenuation of p47phox membrane translocation and phosphorylation. *Journal of Biological Chemistry* **273**, 32801–32805.

Cheeseman KH, Holley AE, Kelly FJ, Wasil M, Hughes L & Burton G (1995) Biokinetics in humans of RRR-alpha-tocopherol: the free phenol, acetate ester, and succinate ester forms of vitamin E. *Free Radicals in Biology and Medicine* **19**, 591–598.

De La Fuente M & Victor M (2000) Anti-oxidants as modulators of the immune system. *Immunology and Cell Biology* **78**, 49–54.

Devaraj S, Li D & Jialal I (1996) The effects of alpha tocopherol supplementation on monocyte function. Decreased lipid oxidation, interleukin 1 beta secretion, and monocyte adhesion to endothelium. *Journal of Clinical Investigation* **98**, 756–763.

Erl W, Weber C, Wardemann C & Weber PC (1997) Alpha-tocopheryl succinate inhibits monocytic cell adhesion to endothelial cells by suppressing NF-kappa B mobilization. *American Journal of Physiology* **273**, H634–H640.

Hidaka H, Inagaki M, Kawamoto S & Sasaki Y (1984) Isoquinoline-sulfonamides, novel and potent inhibitors of cyclic nucleotide dependent kinase and protein kinase C. *Biochemistry* **23**, 5036–5041.

Hidiroglou N, McDowell LR, Papas AM, Antapi M & Wilkinson NS (1992) Bioavailability of vitamin E compounds in lambs. *Journal of Animal Science* **70**, 2556–2561.

Jensen SK, Engberg RM & Hendemann MS (1999) All rac-α-tocopherol acetate is a better vitamin E source than all-rac-α-tocopherol succinate for broilers. *Journal of Nutrition* **129**, 1355–1360.

Kanno T, Utsumi T, Takehara Y, Ide A, Akiyama J, Yoshioka T, Horton AA & Utsumi K (1996) Inhibition of neutrophil-superoxide generation by α-tocopherol and coenzyme Q. *Free Radical Research* **24**, 281–289.

Kim SJ, Bang OS, Lee YS & Kang SS (1998) Production of inducible nitric oxide is required for monocytic differentiation of U937 cells induced by vitamin E-succinate. *Journal of Cell Science* **111**, 435–441.

Kline K & Sanders BG (1993) RRR-alpha-tocopheryl succinate inhibition of lectin-induced T cell proliferation. *Nutrition and Cancer* **19**, 241–252.

Lowe WL, Yorek MA, Karpen CW, Teasdale RM, Hovis JG, Albrecht B & Prokopiou C (1992) Activation of protein kinase C differentially regulates insulin-like growth factor-I and basic fibroblast growth factor messenger RNA levels. *Molecular Endocrinology* **6**, 741–752.

Plesner T, Ploug M, Ellis V, Ronne E, Hoyer-Hansen G, Wittrup M, Pedersen TL, Tscherning T, Dano K & Hansen NE (1994) The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. *Blood* **83**, 808–815.

Politis I (1996) Plasminogen activator system: Implications for mammary growth and involution. *Journal of Dairy Science* **79**, 1097–1107.

Politis I (2000) The role of plasminogen activator in the bovine mammary gland. *Advances in Experimental Biology* **480**, 203–207.

Politis I, Hidiroglou N, Cheli N & Baldi N (2001) Effects of vitamin E on urokinase-plasminogen activator receptor expression by bovine neutrophils. *American Journal of Veterinary Research* **62**, 1934–1938.

Politis I, Hidiroglou N, White JH, Gilmore JA, Williams SN, Scherf H & Frigg M (1996) Effects of vitamin E on mammary and blood leukocyte function, with emphasis on chemotaxis, in periparturient dairy cows. *American Journal of Veterinary Research* **57**, 468–471.

Rego AC, Santos MS, Proenca MT & Oliveira CR (1998) Influence of vitamin E on retinal cell survival. *Toxicology* **128**, 113–124.

Yoshikawa T & Yoshida N (2000) Vitamin E and leukocyte-endothelial interactions. *Antioxidants in Redox Signaling* **2**, 821–825.